Dietary methylmercury alters the proteome in Atlantic salmon (*Salmo salar*) kidney

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Abstract

Background

Methylmercury (MeHg) is an environmental contaminant most known for its severe neurotoxic effects. Although accumulation of MeHg tends to be several folds higher in kidney compared to other tissues, studies on nephrotoxic effects are almost non-existing. In this study we aim to investigate the toxicity of dietary MeHg in kidney of Atlantic salmon (*Salmo salar)*.

Material and methods

Atlantic salmon were exposed to dietary MeHg for a period of 8 weeks, before the fish were euthanized and kidney was sampled for proteomic and real time RT-PCR analysis, as well as for mercury determination. Protein separation was done with 2-D PAGE, and differentially regulated spots were picked for analysis using liquid chromatography MS/MS analysis. Moreover, whole blood and liver tissue were sampled for mercury determination and real time RT-PCR (liver).

Results

MeHg exposed fish accumulated significantly more mercury (Hg) than control fish. The proteomic analysis revealed differential abundance of 26 spots in the kidney, and 14 of these protein spots were successfully identified. The proteins identified indicated effects of MeHg on; metabolism, inflammation, oxidative stress, protein-folding, and cell-structural components. Gene expression analysis of selected markers revealed few differentially regulated transcripts in kidney and liver in the exposed fish compared to the control fish. However, the affected transcripts indicated a disruption in the expression of two metabolic markers due to MeHg exposure in liver.

Conclusion

This study suggests that dietary MeHg has similar effects in kidney as previously shown for other tissues in fish. The effects observed were in markers for oxidative stress, inflammation and energy metabolism. The identification of proteomic markers in this study provides a basis for a better understanding of MeHg-induced nephrotoxicity in fish.

Keywords:

 Methylmercury, kidney, Atlantic salmon, proteomics.

1. Introduction

Methylmercury (MeHg) is an environmental contaminant produced from elemental mercury by different sulphate reducing microorganisms (Jensen and Jernelov, 1969). MeHg easily bioaccumulates, with consequent biomagnification in the marine food chain, and eventually poses a threat to higher trophic layers of fish and mammals through dietary exposure. The LD 50 of MeHg for salmonids has been reported to be as high as 15 mg Hg kg^{-1} in rainbow trout (*Onchorhynchus mykiss*) (Miettinen et al., 1970). However, the effects of MeHg occur at both population and individual level at more environmentally relevant doses. Scheuhammer et al (2007) reviewed the environmental effects of MeHg on wild fish, and MeHg were shown to affect the reproductive capability. Although MeHg shows effects on populations, the molecular responses in individual fish, have been more thoroughly studied in order to understand the molecular toxicology of MeHg in fish. High throughput analyses, such as microarray and proteomic analyses, have been used to determine biomarkers and elucidate toxic modes of action for MeHg in different tissues and species (Berg et al., 2010; Klaper et al., 2008; Richter et al., 2011).

The neurological system has been deemed the main target for MeHg, and neurological effects of MeHg have been investigated in different species. A proteomic study investigating MeHg effects in the brain of Atlantic cod (*Gadus morhua*) after intraperitoneal injections revealed effects on oxidative stress, cell structural degeneration, calcium homeostasis, and on metabolic markers (Berg et al., 2010). Oxidative effects of dietary MeHg in the neurological system have also been observed in Atlantic salmon (Berntssen et al., 2003). The toxic response to MeHg is, however, not limited to the neurological system. A microarray study performed on acute and chronic intraperitoneally injected doses of MeHg in fathead minnow (*Pimephales promelas*), revealed toxic effects in the liver and gonads of the fish (Klaper et al., 2008). Differing effects after acute and chronic exposure in adaptation mechanisms to MeHg were also observed, for example in markers for protein processing (Klaper et al., 2008). In addition, the up-regulation of apoptotic markers was observed, and was suggested as an explanation to the detrimental effects of MeHg on reproduction (Klaper et al., 2008). In skeletal muscle of the zebra fish (*Danio rerio*), MeHg exposure affected mitochondrial energy metabolism by inhibiting the coupled ATP oxidative phosphorylation (Bourdineaud et al., 2009).

Overall, based on current knowledge, MeHg toxicity seems to rely on several molecular mechanisms, depending on the tissue studied. However, to our knowledge, no studies have investigated the effect of MeHg in fish kidney, even though this organ accumulates among the highest amounts of MeHg in salmonids (Berntssen et al., 2003; Boudou and Ribeyre, 1985). In order to investigate mechanisms of the renal-response to MeHg contamination, proteomic analysis (2-D PAGE and HPLC MS/MS) was used to screen for effects in the kidney of Atlantic salmon exposed to MeHg for eight weeks. For a

selected set of the identified proteins responding to MeHg exposure, as well as a few additional MeHg marker proteins, the transcriptional levels were also determined in the kidney by the means of real time RT-PCR. For comparative reasons we investigated the expression of these same genes in the liver, as liver tissue is also regarded as central to detoxification.

2. Material and methods

2.1 Feed production

Experimental diets were prepared based on a basal fish diets at NOFIMA (Titlestad, Bergen, Norway) March 2009. Diet composition per kg was: 505 g fish meal, 60 g wheat gluten, 160 g wheat, 250 g fish oil, 20 g vitamin mix, 4 g mineral mix, 0.13 g yttrium oxide, and 0.5 g astaxanthin (Carophyll Pink®**)**. To ensure a low level of contaminants (mainly lipid soluble contaminants) in the experimental diets, cleaned fish oil was used (FF Skagen, Skagen, Denmark). Two experimental diets were produced; one control diet and one enriched with MeHg. The MeHg were added as methylmercury chloride (PESTANAL®, Sigma-Aldrich, Seelze, Germany) to a nominal concentration of 4 mg Hg kg^{-1} feed. The final Hg concentrations were determined as 3.8 ± 0.2 mg kg^{-1} (n = 3) and 0.07 ± 0.01 mg kg^{-1} (n = 3) in the MeHg enriched diet and non-enriched (control) diet, respectively.

2.2 Feeding trial

The feeding trial was carried out at NOFIMA Aquaculture Research Station (Austevoll, Norway) between March and May 2009. The Atlantic salmon were obtained from Sjøtroll Havbruk AS (Bekkjarvik, Norway). The fish (initial weight 564 ± 131 g, n = 18) were randomly distributed to six glass fibre tanks (2m × 2m) with 50 individuals in each tank. The tanks were supplied with a continuous flow of seawater (80 L/min). The seawater had an average temperature of 9.6 ± 1.0 °C during the feeding trial. The fish were kept under continuous light and fed by automatic feeders (feeding time: 1400-0300 and 0500-0800). The diets were fed to fish in triplicate tanks for eight weeks (54 days).

2.3 Sampling

Fish were collected one day after the end of the feeding experiment, and euthanized with a blow to the head. The length and weight of the fish were recorded, and blood was drawn from vena caudalis into heparinized vials for further Hg analysis and hematocrit measurements. The kidney and liver was excised and flash frozen in liquid nitrogen, before they were stored at -80°C for later analysis.

2.4 Hg analysis

Total Hg was measured in the kidney and liver samples from each individual fish using atomic absorption spectrophotometry (Direct mercury analyzer, DMA-80; Milestone, Sorisole, Italy). The accuracy of the analytical methods was assessed by the analysis of two certified reference materials; oyster tissue (SMR 1566b; National Institute of Standards and Technology, Gaitersburg, MD, USA; certified value 0.0371 ± 0.0013 μg Hg g^{-1} dry weight) and milk powder (BCR 150; Institute for Reference Material and Measurements; Geel, Belgium; certified value 9.4 ± 1.7 ng Hg g^{-1} dry weight)

in each analytical run. The values obtained experimentally were in good agreement with the certified values

2.5 Real time RT-PCR

Total RNA extracted from tissue from both kidney and liver was analyzed using selected genes in real time RT-PCR (Table 2). Tissue was homogenized, and total RNA extracted using RNeasy columns (Qiagen, Oslo, Norway). The quantity and quality of the RNA was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The Bioanalyzer gave a RNA integrity number (RIN) of 9.7 ± 0.1 for kidney (n = 6), and 9.4 ± 0.1 for liver (n = 6).

The expression of target genes in Atlantic salmon exposed to dietary MeHg, was analyzed using a two-step real-time RT-qPCR protocol (Olsvik et al., 2010). The RT reactions were run in triplicates in a 96 well plate, with 500 ng RNA in each sample. For PCR efficiency calculations a 6-fold serial dilution (1000-31 ng) in triplicates, was made from a pooled RNA sample. Template controls (ntc) and RT enzyme control (nac) were included in each 384 well plate. The RT reaction was performed according to the manufacturer`s instructions using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) on a GeneAmp PCR 9700 machine (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2.5 µM) for all genes in 30 µl total volume. The final concentration of the other chemicals in each RT reaction was: $MgCl_2$ (5.5 mM), dNTP (500 mM of each), 10X TaqMan RT buffer (1X), RNase inhibitor (0.4 U/µl) and Multiscribe reverse transcriptase (1.67 U $µl^{-1}$) (Applied Biosystems). Diluted cDNA (1:2) (2.0 µl cDNA from each RT reaction) was transferred to a 384-well reaction plate and the qPCR run in 10 µl reactions on the LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). Real-time PCR was performed by using SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche Applied Sciences,), which contains FastStart DNA polymerase and gene-specific primers (500 nM). PCR was achieved with a 5 min activation and denaturizing step at 95°C, followed by 45 cycles of a 15 s denaturing step at 95°C, a 60 s annealing step and a 30 s synthesis step at 72°C. Target gene mean normalized expression (MNE) was determined using a normalization factor calculated by the *geNorm* software based on selected reference genes (Vandesompele et al., 2002). All genes showed a stable expression both in kidney and liver tissues, i. e. with M-values below 0.5. In order to select the most stabile reference genes available, all genes analyzed were checked for stability using the geNorm and NormFinder software packages. In kidney it was chosen to use elongation factor 1 alpha b (ef1ab) and carnitine palmitoyltransferase 1 (cpt1), as suggested by both the geNorm and NormFinder softwares. In liver, nine genes were chosen: B-cell lymphoma X (bclx), fatty acid binding protein (fabp), calreticulin (crc), heat shock protein 90 (grp94), tubulin alpha (tuba),

annexin 13 (anxa13), beta actin (actb), and ef1ab, according to the suggestion by the NormFinder software.

2.6 Protein extraction and gel analysis

Protein extraction and analysis were performed according to Cash et al (1995). Kidney samples (n=6) were homogenized in 2-D Lysis Buffer (0.5 ml 0.5MTris-HCl pH 6.8, 0.125 ml 0.2M EDTA, 12 g Urea, 2.5 ml 0.5 M DTT, 2.5 ml Glycerol, 1.25 ml NP-40, 3.7 ml pH 3-10 ampholytes, 5 ml MilliQ water) using a pestill at room temperature. Lysis buffer was added in a 10:1 ratio. The cell homogenates were centrifuged at 11,000 x g for five minutes, and the supernatant was decanted and used for further analysis. Proteins from the kidney homogenate were precipitated using ReadyPrep™ 2-D Clean kit (Bio-Rad Laboratories, Hercules USA) according to the manufacturer`s instructions. The precipitate was solubilized in IPG Reswell buffer (2.01 g UREA, 0.76 g Thiourea, 0.2 g CHAPS, 0.015 g DTT, 3 ml MilliQ water, 50 µl pH 4-7 IPG buffer (GE Healthcare) and sufficient bromophenol blue to give the solution a blue colour). The protein solution was sonicated with 3 bursts each of 5 seconds, and then incubated with one part DNase solution (0.05 ml 1M $MgCl_2$, 0.5 ml 1 M Tris-HCl pH 8.0, and 0.1ml 20 000 U ml^{-1}) to two parts protein solution for 10 minutes on ice.

Protein samples were initially analyzed by 1-Dimensional SDS PAGE to check protein quality and determine protein loading for 2D PAGE. Typically, IPG strips (Immobiline™ DryStrip pH 4-7, 7 cm, GE Healthcare, Amersham, UK) were rehydrated overnight with 15 µl sample adjusted to a final volume of 125µl with IPG reswell buffer. First dimension isoelectric focusing was run on a Multiphor II (GE Healthcare) during three steps of different voltage (1 min at 200 V, 1.5 h at 3500 V, and 1 h 5 min at 3500 V). A ramped change in voltage was applied between steps 2 and 3. Then, the IPG gel strips were equilibrated for 30 min in 10 mg ml^{-1} DTT and subsequently 30 min in 25 mg ml^{-1} iodoacetamide (both prepared in an equilibration stock buffer, 0.05 mg ml^{-1} Tris, 6 M UREA, 30 % glycerol, and 10 % SDS), before they were transferred to the second dimension gels (NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels, IPG Well 1.0 mm, Invitrogen, Paisley, UK). The gels were run in an XCell *SureLock*™ Mini-Cell (Invitrogen, Paisley, UK), at 100 V for 1 h and 200 V for 1.5 h in MOPS running buffer (Invitrogen). The gels were fixed (50 % ethanol and 2 % phosphoric acid) over night, before they were rinsed in milliQ water and stained with Comassie Brilliant Blue G-250 in an equilibration buffer (68 ml methanol, 34 g ammonium sulphate, 4 ml ortophosphoric acid, and milliQ water to 200ml total) for up to 4 days.

The stained gels were scanned wet in an ImageScanner™III (GE Healthcare, UK) with LabScan software (GE Healthcare, UK), obtaining 16 bit images, and a resolution of 600 dpi. The images were imported into Progenesis SameSpots, version 4.1 (Nonlinear Dynamics, Newcastle upon Tyne, UK).

For a review on this software see Silva et al. (2010). A reference gel from the control samples was chosen, and the remaining gel images were aligned to this. Spots were detected automatically, and then reviewed manually using the software. Spots showing a significant difference (p ≤ 0.05) between treatments were picked.

2.7 Protein identification by in gel trypsin digestion

Following 2-D gel electrophoresis, the protein spots of interest were excised from the gel. Proteins in the gel pieces were digested with trypsin (sequencing grade, modified; Promega UK, Southampton, UK) using an Investigator ProGest robotic workstation (Genomic Solutions Ltd., Huntingdon, UK). Briefly, proteins were reduced with DTT (60°C, 20 min), S-alkylated with iodoacetamide (25°C, 10 min) then digested with trypsin (37°C, 8 h). The resulting tryptic peptide extract was dried by rotary evaporation (SC110 SpeedVac; Savant Instruments, Holbrook, NY, USA) and dissolved in 0.1% formic acid for LC-MS/MS analysis.

2.8 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

Peptide solutions were analysed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Coventry, UK) coupled to an UltiMate 3000 LC System (Dionex Ltd., Camberley, Surrey, UK). Peptides were separated on a Monolithic Capillary Column (200 μm i.d. x 5 cm; Dionex) at a flow rate of 2.5 μl min^{-1} using a gradient of acetonitrile (6 – 38% over 12 min) in 0.04% (aq.) formic acid. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300 – 1500 m z^{-1}, 3 averages, and up to 3 precursor ions selected from the MS scan (100 – 2200 m z). Precursors were actively excluded within a 1.0 min window, and all singly-charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using DataAnalysis software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS Ions searches of the NCBInr database using the Matrix Science web server (www.matrixscience.com). The default search parameters used were: Enzyme = Trypsin; Max. missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP.

2.9 Functional annotation and pathway analysis

The proteins identified were functionally annotated using www.genecards.com. The letter codes obtained from www.genecards.com correspond to mammalian proteins, so additional NCBI blast searches were used as confirmation of similarity between the fish protein, and the corresponding mammalian protein. The proteins and their fold change were analyzed using "Tox analysis" (limited to kidney) in Ingenuity Pathway Analysis (IPA).

2.10 Statistical analysis

Differences in the physiological parameters of the fish (weight, length, mortality and hematocrit), were analyzed using student t-test ($p < 0.05$), and presented as mean ± SD. Mercury analysis and gene expression statistics were investigated using Statistica 9 (StatSoft Inc., Tulsa, USA). Differences in gene-expression were analyzed using the non-parametric Mann-Whitney U test. Differences in protein spots were detected with one way ANOVA using Progenesis SameSpots. All analyses were checked for tank effects using one-way ANOVA, or Kruskal-Wallis. Effects observed in all analyses were deemed significant when $p < 0.05$.

3. Results

3.1 MeHg accumulated in tissues, but did not affect physiological parameters of the fish

MeHg did not significantly affect mortality (no mortality observed), nor growth of the Atlantic salmon as measured by final length (43.9 ± 1.9 cm, n = 18) and weight (1240 ± 185 g, n = 18). No significant differences were observed in the blood parameter hematocrit (38 ± 8 %, n = 14) of the exposed salmon ($p < 0.05$) compared to control fish. A significant accumulation of MeHg was seen in plasma, red blood cells, liver and kidney of the exposed fish (Table 1). When comparing the accumulation in the different tissues, high to low accumulation were observed in the respective order: Red blood cells > liver ≈ kidney >> plasma.

3.2 Dietary MeHg affected proteomic abundances in kidney of salmon

Protein abundances from kidney samples were compared through 2-D gel analysis using Progenesis SameSpots. The gels showed good resolution (fig. 1) and after editing gels for background, 468 spots were detected and included in the analysis. From these, 26 spots were significantly regulated ($p < 0.05$) in response to MeHg. 18 of these spots were selected for identification using peptide fragment fingerprinting and LC MS/MS, and from these 14 were successfully identified (Table 3). All proteins identified showed low fold changes, but were all significantly up- or down-regulated. Alpha-1-antiproteinase-like protein (SERPINA7), hemopexin like protein (HPX), and vacuolar protein sorting associated protein 29 (VSP29) were identified in two or three different spots. This was probably due to the occurrence of post translational modifications (PTMs) or degradation of the protein, where both of these factors can affect protein isoelectric point (PI) or molecular weight. Otherwise, all proteins theoretical pI and molecular weights were consistent with the observed electrophoretic mobilities by 2D-PAGE. In addition to the aforementioned proteins, the following proteins were successfully identified: β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione peroxidase 2 (GPX2), heat shock 90 protein 1 β isoform a (Hsp90AB1), 60 kDa heat shock protein (HSPD1), galectin 2 (LGALS2) and ATP synthase H$^+$ transporting mitochondrial F1 complex β (ATP5B).

3.3 MeHg showed minor effects on transcriptomic data.

Proteins identified in this study, and proteins regulated by MeHg and fatty acids in another study (Nøstbakken et al., In prep.), were used as a starting point for primer design when investigating the transcriptional response of Atlantic salmon kidney and liver to MeHg. These, and certain transcripts otherwise known to be affected by MeHg, were analyzed in kidney and liver from the MeHg exposed Atlantic salmon and compared to control fish. Due to the relatively small changes observed in gene expression, the choice of reference genes was determined after evaluating the stability of all analyzed genes. This also affected the range of target genes analyzed in the different tissues. In

kidney, the following target genes were analyzed: fatty acid binding protein 3 (fabp3), glutathione peroxidase 2 (gpx2), acetyl-co-oxidase (aco), aldo-keto reductase 1B3 (akr 1b3), 14-3-3E1, annexin A13 (anxa13), glyceraldehyde-3-phosphate dehydrogenase (gapdh), ferritin, tubulin α (tuba), heat shock protein 90kDa beta (grp94), calreticulin (crc), B-cell lymphoma X (bclx), warm temperature acclimation related 65 kDa protein/hemopexin (wap65), beta actin (actb), actin related protein (arp) and ubiqutin (ubi) (Table 4). No significant effects were observed in any of the genes analyzed in kidney. In liver, the following target genes were analyzed: fatty acid transport protein (fatp), gpx2, ferritin, wap65, gapdh, 14-3-3b1, akr 1b3, aco, and arp (Table 4). In liver aco and gapdh showed a significant decreased gene expression in MeHg-exposed fish ($p < 0.05$) (fig. 2).

3.4 Pathway analysis

Proteins differentially regulated by MeHg were analyzed using Ingenuity Pathway Analysis (IPA). Pathway analysis incorporated the differentially regulated proteins in a network with a score of 11. Proteins in this network is involved in; carbohydrate metabolism, energy production, and, nucleic acid metabolism. IPA revealed that the top toxicological pathways associated with MeHg in this study were: oxidative stress, increased renal proliferation, hypoxia, gene regulation of Peroxisome Proliferator-activated receptor (PPARα), and mitochondrial dysfunctions. Additionally, several biological functions were related to the differentially regulated proteins in this study (Supplementary data Table).

4. Discussion

Few studies have investigated the molecular response induced by MeHg in the kidney of fish, even though the kidney accumulates relatively high amount of MeHg in salmonids (Berntssen et al., 2003; Boudou and Ribeyre, 1985), even under sub-lethal conditions. A study comparing effects of MeHg on oxidative stress and tissue damage in Atlantic salmon parr, revealed more detrimental effects in nervous tissue than in kidney and liver, although accumulation of MeHg in kidney and liver were higher (Berntssen et al., 2003). Compared to the analyses by Berntsen et al (2003), similar accumulation of MeHg in kidney and liver was observed in our study. To further explore MeHg-induced molecular responses in the Atlantic salmon kidney, we implemented exploratory proteomics in our study. MeHg affected proteomic markers involved in oxidative stress, metabolic toxicity, inflammation, and protein folding and handling. These effects were similar to those observed in other fish studies (Berg et al., 2010; Klaper et al., 2008; Richter et al., 2011), implying that Atlantic salmon kidney showed similar modes of toxicity as in other tissues. However, all fold changes in protein abundance in our study indicated a lower response in the kidney compared to tissues studied in other proteomic studies (Berg et al., 2010). However, some aspect of the low fold changes in protein abundance that we observed compared to others may be due to the choice of species, route of administration and methodological approaches. In addition to the proteomics data, transcriptional changes of selected gene markers were assessed. Transcriptional regulation was in general shown to be low in both kidney and liver, which could be attributed to the long term aspect of this study. In fact, the transcriptional responses were so marginal that α-tubulin (tuba), a known marker of structural degradation caused by MeHg (Crespo-López et al., 2009), was suggested as a reference gene by both the geNorm and NormFinder reference gene selection softwares, due to its stabile expression. As a result, the main focus in this discussion was based on the proteomic data.

4.1 Dietary MeHg induce oxidative stress in the kidney

A well known toxic effect of MeHg is its ability to induce oxidative stress (Aschner et al., 2007; Berntssen et al., 2003). The influence of MeHg on the red-ox systems in cells has been largely attributed to the production of reactive oxygen species (ROS) (Ni et al., 2010), and/or inhibition of antioxidant systems, such as the glutathione system (Gatti et al., 2004). Our proteomic study revealed that GPX2 was increased in abundance in the kidney of MeHg- exposed fish compared to control fish. This was consistent with findings by Cuello et al. (2010), where an increased activity of GPX was observed in the hepatic cell line HepG2, after MeHg exposure. However, several studies have reported that GPX-activity is down-regulated in response to MeHg (Franco et al., 2009; Glaser et al., 2010). GPXs are a family of enzymes capable of reducing several organic and inorganic hydroperoxides, and thereby reducing oxidative stress in cells (Masella et al., 2005). As GPX are

selenium dependent enzymes (Rotruck et al., 1973), their activity is dependent on the availability of selenium. MeHg can post-trancriptionally affect the activity of GPX through the binding of free selenium, or direct binding of the active site of GPX thereby blocking its function (Ralston et al., 2008). The increased protein abundancy of GPX observed in our study may be the kidneys transcriptional response to oxidative stress, without necessarily increasing the enzymatic activity of GPX. Still, it remains an indication of increased oxidative stress in the kidney. HPX, another marker for oxidative stress (Li et al., 2009), was significantly down-regulated in kidney of the MeHg-exposed fish. No effect was, however, observed on the transcriptional levels of the warm-temperature-acclimation-associated protein (wap65), a plasma glycoprotein showing homology to mammalian hemopexin (Sarropoulou et al., 2010). The human homologue to fish HPX; hemopexin (HPX) has previously been deemed as a biomarker for nephrotoxicity (Thukral et al., 2005). However, the faltered protein abundance of fish HPX, may be more indicative of MeHg-induced blood physiological changes in the fish. Mammalian HPX is a hematological protein, and probably stems from blood within the kidney at the time of excision. The main function of HPX is binding and exporting heme from the blood to the liver, clearing the blood of excess iron after hemolysis and recirculation of heme for hematopoesis (Tolosano et al., 2010). In a study using hemopexin-deficient mice, an increase in iron deposition in the kidney, and consequent nephrotoxicity due to increased oxidative stress in the kidney, was observed (Tolosano et al., 1999). In our study red blood cells accumulated high amounts of Hg, and MeHg-induced hemolysis of red blood cells with consequent iron deposition in the liver may be another mechanism explaining the elevated oxidative stress in the kidney.

4.2 Dietary MeHg affects energy metabolism

The production of ROS, and consequent oxidative stress, has been explained as a secondary response in MeHg toxicity due to MeHg-induced effects on electron transfer in mitochondrial energy metabolism (Yee and Choi, 1996). There exist several studies showing molecular effects of MeHg on metabolic markers (Berg et al., 2010; Cambier et al., 2009; Thukral et al., 2005). Thukral et al (2005) showed a reduced expression of genes associated with β-oxidation, electron transfer, fatty acid transport and nitrogen metabolism in the kidney of rats after inorganic Hg exposure. These effects were regarded as indicative of renal toxicity since renal secretion is an active process (Thukral et al., 2005). In our study, ATP5B was down-regulated in MeHg exposed fish. MeHg has been shown to inhibit the rate III mitochondrial respiration (ADP- dependent oxidative phosphorylation), but not the rate IV mitochondrial respiration in zebra fish skeletal muscle (Bourdineaud et al., 2009). This implies that MeHg may inhibit the enzymes responsible for phosphorylation of ATP in the mitochondria, such as ATP5B. Moreover, GAPDH, an important catalyst in the energy-yielding step of carbohydrate

metabolism, was down-regulated in our study, suggesting effects of MeHg on mitochondrial energy metabolism.

Compared to the proteomic data, gene transcriptional analysis revealed few effects of MeHg. In kidney, out of a total of 16 genes analyzed, no significant transcriptional response were observed. In liver, however, the expression of aco and gapdh were expressed significantly lower in response to MeHg. Both genes are involved in energy metabolism, suggesting that MeHg may affect metabolic processes at transcriptional and/or post-transcriptional levels. The most noteworthy regarding the transcriptomic results, however, was the relatively stable expression observed for most transcripts. This indicated that transcriptomic alterations were less suited as biomarkers during long-term MeHg exposure than responses at the protein level, underlining the importance of investigating a broad range of molecular parameters. The fact that the liver also displayed a relative stable transcriptional expression, suggests toxicity adaptation in the organism as a whole.

4.3 MeHg affects immunological response

Compared to the effects on metabolic and oxidative stress, much less is known about the effects of MeHg on inflammation. The kidney is an important immunological organ in teleost fishes (Iwama et al., 1996), and, together with blood, have a higher abundance of immune related cells compared to other tissues normally investigated for MeHg toxicity, such as brain, liver or muscle. Therefore, the kidney and blood may provide a more sensitive response for immunological markers in our proteomic study. An effect of MeHg exposure was seen on the immune-related proteins LGALS2 and SERPINA7, possibly linking MeHg-exposure to immunological responses in Atlantic salmon kidney. In addition, differential regulation of the aforementioned HPX may be indicative of an immunological response (Bakker et al., 2010). Galectins are highly conserved in evolution (Rabinovich et al., 2002), confirmed by Blastx search which showed that the peptide sequence annotated to LGALS2 from salmon kidney had a 96% similarity against the human galectin 2 (LGALS2) protein. Human LGALS2 is normally expressed in the gastrointestinal system and in some tumors, and has been linked to the induction of apoptosis in T-cells (Sturm et al., 2004). Galectins show a high sequence similarity in the carbohydrate recognition domain (CRD) (Rabinovich et al., 2002), possibly indicating that the identified LGALS2 from salmon kidney may be homologous to another isoform of the galectin superfamily since the main hits in our Mascot search were in the CRD (controlled by ExPASy search). Either way, LGALS2 was differently regulated in kidney of the MeHg exposed fish, suggesting an immunological effect.

The SERPINA7 in salmonids was first identified in rainbow trout seminal plasma (Mak et al., 2004). This protein showed high similarity to the mammalian serine protease inhibitor (serpin) family which

are plasma proteins involved in several regulatory functions (Potempa et al., 1994). The main mammalian serine protease inhibitor in blood is the α-1-antiproteinase inhibitor (the equivalent version to the rainbow trout SERPINA7) of which the main function is to inhibit neutrophil elastase and thereby playing a possible anti-inflammatory role. In our study we identified a down-regulation of SERPINA7, indicating an inflammatory response to MeHg.

4.4 Conclusion

This is the first study investigating toxic effects of MeHg in the proteome of the Atlantic salmon kidney. MeHg increased the abundance of proteins related to oxidative stress in the kidney, as indicated by the increased abundance of GPX2 at the protein level. Several proteins involved in energy metabolism, especially mitochondrial energy metabolism, showed decreased abundance in response to MeHg exposure both at the transcriptional and the proteomic level. Oxidative stress and reduced energy metabolism are well-known toxicological markers of MeHg exposure, however, immunological effects have been less affiliated with MeHg treatment. The observed immunological effect of MeHg may be organ-dependent, and should be examined further in follow-up studies.

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Figure captions

Figure 1

Representative 2-D gel. The positions of the spots excised from the gel are indicated by circle. Identities of these proteins are shown by lettering.

Figure 2

Mean normalized gene expression (MNE) of: (A) gapdh and (B) aco in liver from Atlantic salmon. Data represented by median, with boxes representing interquartile range, and whiskers representing total range. Significant differences are indicated through small letters ($p < 0.05$). Statistic analysis performed using the non-parametric Mann-Whitney U test.

Tables

Table 1. Hg content (mg kg^{-1}) of fish exposed to 4 mg kg^{-1} MeHg in feed and control for 8 weeks.

	MeHg exposed fish	Control
Kidney	2.96 ± 0.44 [a]	0.10 ± 0.02 [b]
Liver	4.18 ± 1.19 [a]	0.06 ± 0.04 [b]
Red Blood cells	7.12 ± 3.33 [a]	0.11 ± 0.05 [b]
Plasma	0.21 ± 0.16 [a]	0.01 ± 0.02 [b]

Data presented as median ± range. In kidney samples (N = 16), in liver (N =18), significant differences are indicated though superscript small letters ($p < 0.05$). Data were tested by non parametric Kruskal Wallis test and post hoc paired comparisons.

Table 2. Q-PCR Primer pair information, all sequences are presented as 5´ to 3´.

Gene	Acession	Fwd primer	Rev. Primer	amplicon size	PCR efficiency
ARP	AY255630	GAAAATCATCCAATTGCTGGATG	CTTCCCACGCAAGGACAGA	101	2.08[a], 1.93[b]
EF1Ab	BG933853	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	59	2.20[a], 1.89[b]
ACTB	NM_001124235	GCCGCGACCTCACAGACTAC	CAAAGTCCAGCGCCACGTA	126	2.12[a], 2.09[b]
Ubi	BG936428	GATCTTCGCTGGCAAACAACT	CGAAGACGCAGCACAAGATG	93	2.01[a], 1.94[b]
FATP	CA373015/ AF023258	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTGG	64	X[a], 2.19[b]
ACO	DQ364432	CACTGCCAGGTGTGGTGGTA	GGAATTGTACGTTCTCCAATTTCA	92	2.06[a], 2.02[b]
FABP3	AY509548	CACCGCTGACGACAGGAAA	TGCACGTGAACCATCTTACCA	66	1.93[a], 1.97[b]
TUBA	BT049768	GAGCCAGCCAATCAGATGGT	TGCGCTTGGTCTTGATTGTG	110	2.05[a], 1.98[b]
GSH-PX	BQ036408	TTCTCCACCACACTGGGATCA	GGAAATGGCATCAAGTGGAATT	101	2.07[a], 1.89[b]
CPT1	AM230810	CTTTGGGAAGGGCCTGATC	CATGGACGCCTCGTACGTTA	121	1.98[a], 1.98[b]
Bcl-X	NM_001141086	GCCTGGACGCAGTGAAAGAG	GGACGGCGTGATGTGTAGCT	107	1.10[a], 2.16[b]
CRC	BT044674	CACCAAGAAGGTTCATGTCATC	GTCTGGTCCGGGTTCAGGAT	123	2.08[a], 2.11[b]
Akr1B3	BT049670	TCTCTGCTCGAGGACCCAAA	CGTAGCCGACTTGGGAATGA	120	2.08[a], 1.99[b]
GRP94	NM_00119500	CTGGGTACCATCGCCAAGTC	CAACCAGGAAGGCGGAGTAG	130	2.19[a], 2.19[b]
Ferritin	BT046912	CCGGATGATCAACATGGAGAT	TCGTCGCTGTTCTCCTTGAA	120	2.00[a], 1.87[b]
14-3-3E1	BT045507	GATCCGGGAATACAGGCAAA	GACCTTGGACTCTCCCGTGTT	115	2.02[a], 2.05[b]
ANXA 13	BT044880	GGGTCTGGCTGAAGCAGATG	AGGTGGCCTGTAGCTGAAGGT	122	2.17[a], 2.14[b]
WAP 65	Z68112	GAGGTGGATGCCACCTTCTC	TCCCAGCACCTCCTTCAAAG	132	2.02[a], 2.02[b]
GAPDH	BT045621	CCACTCCATCTCCGTATTCCA	TGGGAAGAGGCCTTGTCAAT	120	2.02[a], 2.12[b]

X denotes a non functional assay , superscript a denotes PCR efficiency in kidney, and superscript b denotes PCR efficiency in liver

Table 3. Identification of significantly regulated proteins by LC MS/MS.

Letter code[a]	identification	fold change	Score[b]	kDa[c]	PI[c]	Species	Seq. Coverage (%)[d]	Matches[e]	Sequences[f]	emPAI[g]
SERPINA7	alpha-1-antiproteinase-like protein	-1.3	63	47.67	5.86	O. mykiss	2	2 (1)	1(1)	0.07
SERPINA7	alpha-1-antiproteinase-like protein	-1.3	63	47.67	5.86	O. mykiss	2	4(1)	1(1)	0.07
ACTB	beta actin	-1.3	615	42.01	5.38	O. mykiss	39	28(2)	14(1)	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-1.3	423	36.56	6.07	Salmo salar	29	16(4)	8(4)	0.68
GPX2	glutathione peroxidase 2	1.2	136	16.53	5.93	O. mykiss	39	5(0)	5(0)	
VSP29	vacuolar protein sorting associated protein 29	-1.2	259	20.6	6.22	Salmo salar	40	11(1)	8(1)	
VSP29	vacuolar protein sorting associated protein 29	-1.3				Salmo salar				
HSP90AB1	heat shock 90 protein 1 beta isoform a	-1.3	150	83.61		Salmo salar	6	5(1)	4(1)	
HPX	hemopexin like protein	-1.3	126	51.11	5.61	O. mykiss		3(1)	2(1)	0.06
HSPD1	60 kDa heat shock protein	-1.2	193	61.33	5.62	Salmo salar	18	7(0)	6(0)	0.05
LGALS2	galectin 2	1.2	197	14.88	5.93	Salmo salar	52	6(1)	4(1)	1.28
HPX	hemopexin like protein	-1.5	155	51.11	5.61	O. mykiss	5	5(1)	2(1)	0.06
HPX	hemopexin like protein	-1.4	139	51.1	5.61	Salmo salar	8	3(1)	3(1)	0.06
ATPB5	ATP synthase H+ transporting mitochondrial F1 complex beta	1.1	67	52.91	4.87	Salmo salar	10	3(0)	3(0)	

The superscripts given in the table denotes:

[a] Code given to each protein is based on human based IDs obtained from www.genecards.com, these identities were used as input in the pathway analyses.

[b] Scores are generated through automatic calculation by the mascot search engine based on MOWSE scores (Pappin et al., 1993), scores greater than 60 in the NCBI database are deemed significant (p < 0.05).

[c] Both weight (kDa) and PI of the proteins are given as theoretical values provided from the NCBI database.

[d] Percentage of the sequence covered by identified peptides.

[e] Matches indicates the number of MS/MS spectra matched to this protein (numbers in brackets denote significant matches).

[f] Sequences, count of distinct peptides matched (numbers in brackets denote significant sequences).

[g] Exponentially Modified Protein Abundance Index (emPAI) is a relative quantitation of the protein in the specific spot (Ishihama et al., 2005).

Table 4. Mean normalized gene expression (MNE) and range (presented in brackets) of transcripts analyzed in kidney and liver of Atlantic salmon.

Gene	Kidney		Liver	
	Ctr	MeHg	Ctr	MeHg
GPX2	0.65 ± (0.28)	0.72 ± (0.21)	0.58 ± (0.85)	0.65 ± (0.60)
Ferritin	0.78 ± (0.39)	0.89 ± (0.38)	0.81 ± (0.83)	0.78 ± (0.20)
WAP 65	0.64 ± (0.46)	0.63 ± (0.48)	0.57 ± (0.50)	0.55 ± (0.36)
GAPDH	0.63 ± (0.39)	0.71 ± (0.38)	0.67 ± (0.44)*	0.50 ± (0.39)
14-3-3E1	0.69 ± (0.44)	0.70 ± (0.27)	0.78 ± (0.23)	0.85 ± (0.55)
Akr1B3	0.54 ± (0.93)	0.40 ± (0.20)	0.46 ± (0.75)	0.34 ± (0.38)
ACO	0.57 ± (0.51)	0.51 ± (0.37)	0.54 ± (0.48)*	0.38 ± (0.50)
ARP	0.66 ± (0.43)	0.68 ± (0.22)	0.55 ± (0.39)	0.41 ± (0.33)
ANXA 13	0.57 ± (0.43)	0.64 ± (0.27)	Ref gene	Ref gene
GRP94	0.67 ± (0.25)	0.73 ± (0.28)	Ref gene	Ref gene
TUBA	0.76 ± (0.19)	0.80 ± (0.38)	Ref gene	Ref gene
FABP3	0.79 ± (0.25)	0.70 ± (0.47)	Ref gene	Ref gene
CRC	0.72 ± (0.34)	0.75 ± (0.60)	Ref gene	Ref gene
BclX	0.75 ± (0.24)	0.75 ± (0.49)	Ref gene	Ref gene
ACTB	0.56 ± (0.31)	0.64 ± (0.52)	Ref gene	Ref gene
Ubi	0.76 ± (0.22)	0.79 ± (0.27)	Ref gene	Ref gene
FATP	N. D.	N. D.	0.70 ± (0.43)	0.52 ± (0.65)

* denotes significantly regulated transcripts (the significantly differentially regulated transcripts are also shown in figure 2) N. D. denotes not detected in q-PCR.

Supplementary table. Top biological functions affected on a proteomic level by MeHg in kidney of Atlantic salmon

IPA tox analysis

Top networks	Score[a]
Carbohydrate metabolism, energy production, nucleic acid metabolism	11
Cellular function and maintenance, embryonic development, tissue morphology	2

Top Tox Lists

Name	p-value[b]	Ratio[c]
NRF2-mediated Oxidative Stress Response	4.06E-03	0.013
Increases renal proliferation	2.13E-02	0.032
Hypoxia-Inducible Factor signaling	3.62E-02	0.019
Mechanism of gene regulation by peroxisome proliferators via PPARα	5.48E-02	0.012
Mitochondrial Dysfunction	6.54E-02	0.01

Top bio functions

Name	p-value[b]	Molecules[d]
Diseases and Disorders		
Inflammatory disease	6.92E-04 - 1.99E-02	2
Neurological disease	6.92E-04 - 3.21E-02	3
Ophtalmic disease	6.92E-04 - 6.92E-04	1
Infection mechanism	1.38E-03 - 1.51E-02	3
Inflammatory response	2.08E-03 - 3.08E-02	1
Molecular and cellular functions		
Protein synthesis	3.42E-04 - 9.66E-03	2
Post-translational modification	5.21E-04 - 6.91E-03	2
Protein folding	5.21E-04 - 6.91E-03	2
Cellular function and maintenance	6.24E-04 - 3.15E-02	4
Carbohydrate	6.92E-04 - 2.06E-02	1
Physiological system development and function		
Organ development	1.38E-03 - 3.15E-02	1
Reproductive system development and function	1.38E-03 - 3.15E-02	1
Hematological system development and function	2.08E-03 - 3.08E-02	2
Immune cell trafficking	2.08E-03 - 3.08E-02	1
Cardiovascular system development and function	4.15E-03 - 1.38E-02	1

Letters in superscript annotate:

a: Score denotes how well a given network relate to the proteins in question. Networks indicates what other proteins could be affected by the proteins regulated in the study, and what biological mechanisms this can be related to.

b: p-value in IPA is calculated using right tailed Fishers Exact test. The p-value indicates how likely it is that associations between these proteins happen by chance.

c: ratio is calculated by dividing network proteins on the number of total proteins in that particular toxic pathway

d: molecules denotes how many proteins are involved in this specific function.
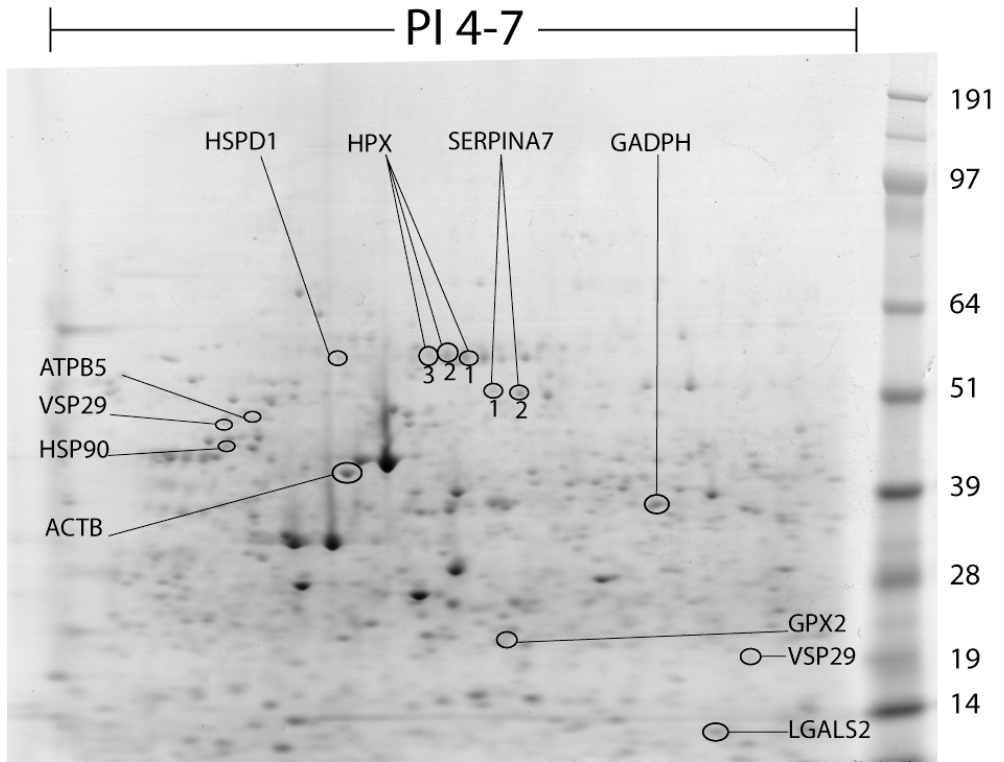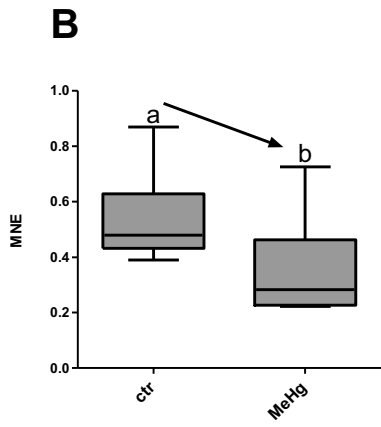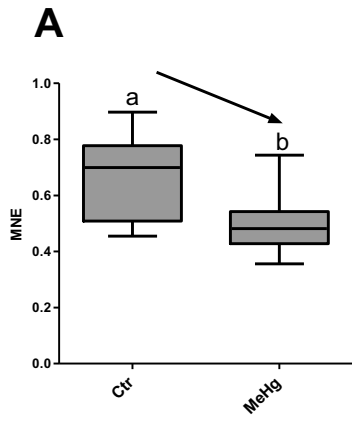
Figure 1

A

B

Figure 2